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EDITORIAL COMMENTARY

Stem cell support of oogenesis in the human†

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The possibility that women produce new oocytes post-natally as part of the normal physiological function of the ovary is currently under investigation. Post-natal production of oocyte-like cells has been detected under experimental conditions in the mouse. Although these cells have many characteristics of oocytes, their potential to mature to fertilization-competence was unproven. Zou *et al*. (Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nat Cell Biol* 2009;**11**:631–636) made use of a striking cell isolation and culture strategy to establish cultures of proliferative germ cells from both newborn and adult ovaries. Their cells, referred to as female germline stem cells (FGSCs), proliferate long-term in culture and accept and maintain expression of a transgenic marker, green fluorescent protein. When delivered to the ovaries of conditioned mice, transgene-bearing FGSC engrafted, were enclosed within follicles, and when host females were mated, transgenic offspring were produced. That proliferative female germ cells capable of giving rise to offspring were detected in adult ovaries poses the question of whether they have a physiological role. Here, we discuss Zou *et al*.'s data in terms of our current understanding of mouse ovarian physiology, and how this may relate to human reproductive biology and the treatment of ovarian dysfunction.

Key words: ovary / oogenesis / stem cells regenerative medicine / menopause

Introduction

In mammals, the production of mature, fertilization-competent oocytes occurs in a remarkably diverse manner (Edwards *et al*., 1977; Jensen *et al*., 2006). The question of whether any mammals produce new eggs during post-natal life has long been a subject of vigorous experimentation and debate. Early in the last century, several authors reported evidence that rats (Arai, 1920), mice (Parkes *et al*., 1927) and rabbits (Pansky and Mossman, 1953) all can produce new oocytes and regenerate lost ovarian tissue [summarized in a contemporary review by Everett (1945)]. It was further shown that oogenesis continues unabated during adult life in some species of prosimian primates (David *et al*., 1974), with the stages of meiotic entry clearly visible in histological preparations of their ovaries. However, a consensus arose in the middle of the last century that humans and the most well-studied domestic and laboratory animals are endowed with their entire complement of oocytes at birth (Zuckerman, 1951).

The question of 'neo-oogenesis' received renewed attention in this century when it was shown that the mouse ovary has an unexpected ability to regenerate immature oocytes after their destruction (Johnson *et al*., 2004; Lee *et al*., 2007). It was shown that both ovary resident (Johnson *et al*., 2004) and circulating bone marrow-derived (Johnson *et al*., 2005a) stem cells can give rise to new immature oocytes in the ovary. A counter-example was published soon thereafter showing that no mature, ovulated oocytes derived from transplanted cells were produced after bone marrow transplantation (Eggan *et al*., 2006). Several reviews discuss the historical ebb and flow in the field (Johnson *et al*., 2005b; Tilly and Johnson, 2007), and indeed, the contentiousness (Telfer *et al*., 2005; Begum *et al*., 2008) of these data.

Here, we consider a recent manuscript in Nature Cell Biology that reports a novel technical and conceptual advance in this area. Kang Zou and co-authors from the School of Life Sciences, Shanghai Jiao Tong University, Shanghai, China, reported that proliferative ovary-resident cells, termed female germline stem cells (FGSCs), cannot only give rise to immature oocytes but can produce new mature, fertilizable oocytes that can produce offspring *in vivo* (Zou *et al*., 2009). Their work is a significant challenge to the dogma that new fertilization-competent oocytes cannot be produced after birth in mammals. Here, we first consider the group's methodology, data and conclusions, placing their work into context with the previous

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literature. Finally, we ask whether these findings are relevant to the function of the human ovary as we consider its physiology and ways to support healthy function and fertility.

Isolation and long-term culture of proliferative female germ cells from post-natal ovaries

Zou *et al*. first sought to confirm the presence of putative FGSCs in young (5-day-old) and adult mouse ovaries. As oocytes are non-proliferative by definition, any cells that express germ lineage markers but are still proliferating would be candidate FGSCs. In keeping with previous studies (Johnson *et al*., 2004), Zou *et al*. detected ovarian cells within the ovarian surface epithelium that were double-positive for Mouse Vasa Homolog (MVH) and 5'-bromodeoxyuridine (BrDU) incorporation. Cells were detected 1 hr after BrDU injection of both 5-day-old and adult mice. Further morphological and histological analysis of the cells was performed, and the authors went on to attempt isolation of these cells for culture *in vitro*. They used an interesting immunomagnetic cell sorting approach targeting predicted surface expression of MVH protein.

MVH is a RNA helicase of the DEAD-box family (Linder, 2006). The Drosophila orthologue, Vasa, acts as a translational regulator of mRNAs localized in oocytes, and is required for proper axis determination in offspring as well as establishment of germ cells (Styhler *et al*., 1998; Tomancak *et al*., 1998; Mahowald, 2001; Riechmann and Ephrussi, 2001). Mutations in the helicase domain of Vasa directly result in germ line defects and female sterility in the fly (Lasko and Ashburner, 1990; Styhler *et al*., 1998). As it is highly expressed in the germ cells of both male and female mammals, MVH is a common molecular marker used for their identification. MVH expression is required for spermatogenesis and thus male fertility (Tanaka *et al*., 2000) in the mouse. In the female, MVH is expressed in the female germline from late primordial germ cell (PGC) migration through the mature metaphase II oocyte (Toyooka *et al*., 2000). The protein is expressed at extremely high levels in the cytoplasm, allowing for excellent signal-to-noise in labeling and cell-tracking experiments.

A pivotal part of Zou *et al*.'s work is the detection of MVH protein on the external surface of the plasma membrane of rare (50–100 cells in the ovaries of 6–8 mice) ovarian cells. In Supplementary Data, they reference the use of the bioinformatics tool TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) to analyze the amino acid sequence of MVH for potential transmembrane/surface domains. They state that two transmembrane domains were predicted by the tool. This justified attempts to target MVH as a potential surface marker in an immunomagnetic cell sorting strategy after enzymatic digestion of ovaries; cells that expressed MVH on their surface were thus separated from cells that lacked surface MVH expression.

The detection of MVH at the surface of germ cells was surprising as this had not been previously reported for any of the highly conserved Vasa orthologues, from flies to man. We used multiple protein sequence analysis tools, including TMPRED, and also found that predicted transmembrane domains of MVH could be identified (not shown). However, these predicted transmembrane domains overlap with two well-characterized functional motifs in the conserved DEAD-box RNA helicase portion of MVH, MVH motifs II and V. These motifs are both known to participate in RNA helicase activity, including ATP, RNA and intra-protein interactions (Linder, 2006; Sengoku *et al*., 2006). It is difficult to understand how these catalytic and binding motifs can simultaneously act as plasma-membrane-spanning domains. More information is needed about the surface MVH immunogenicity of FGSC and whether other germ- or stem-cell markers are expressed on the surface of FGSC.

Questions about surface immunogenicity aside, Zou *et al*. were able to establish cultures of cells isolated using their technique that had remarkable long-term passage characteristics. They showed that freshly isolated and cultured 'FGSC' were proliferative, and confirmed their expression of MVH using immunostaining and the expression of eight additional germ cell (and stem cell-below) specific genes. FGSC did not express markers of either meiosis [Scp1-3 (Yuan *et al*., 2002)], more general oocyte development [e.g. Dazl (Ruggiu *et al*., 1997), Figla (Joshi *et al*., 2007) or Dpa3/Pgc7/Stella (Bortvin *et al*., 2004) or the ZP3 zona pellucida transcript (Lira *et al*., 1990)]. FGSC isolated from newborn mice (nFGSC) and adults (aFGSC) grew in clusters and were able to be cultured on STO feeder cells for more than 15 and 6 months, respectively.

The long-term growth in culture supported experiments that assessed the stem cell properties, or, 'stemness' of FGSCs. nFGSC were found to express Oct4 (Scholer *et al*., 1990; Brehm *et al*., 1998) and Nanog (Chambers *et al*., 2003), and to have high telomerase activity, all characteristics of stem cells.

Importantly, nFGSC were also shown to have a normal karyotype after extended passages. This combination of the expression of 'stemness' related factors (along with the detected germ cell gene expression) and genomic stability suggested to the authors that these cells might be coaxed to support oogenesis.

The production of offspring from ovary-derived FGSCs

Zou *et al*. addressed the question of whether oocytes could be produced using FGSC with a direct *in vivo* approach. In order to track their cells, they indelibly labelled nFGSC and aFGSC using a retroviral vector bearing green fluorescent protein (GFP). The treatment of mice with the chemotherapeutic agents busulfan and cyclophosphamide depletes ovaries of oocytes (Shiromizu *et al*., 1984; Johnson *et al*., 2004). Animals conditioned in this manner have been used as cell recipients in attempts to produce oocytes, with varying results (Eggan *et al*., 2006; Lee *et al*., 2007).

Published reports have only shown the production of low numbers of donor-derived immature oocytes (~0.1% of all oocytes in recipients) when bone marrow- or peripheral blood-derived stem cell fractions were delivered to the bloodstream (Lee *et al*., 2007). Instead, Zou *et al*. delivered their cells directly into the ovaries of recipient animals using pulled glass pipettes. Very strikingly, follicles of all sizes, including large pre-ovulatory follicles, containing GFP-positive oocytes were seen in whole mounts and histological preparations 2 months after delivery of nFGSC or aFGSC (Fig. 1A). In fact, nearly all oocytes shown in fluorescence photomicrographs are unambiguously GFP-positive. The authors reported that oocytes [and] follicles were not seen in control conditioned animals that did not receive

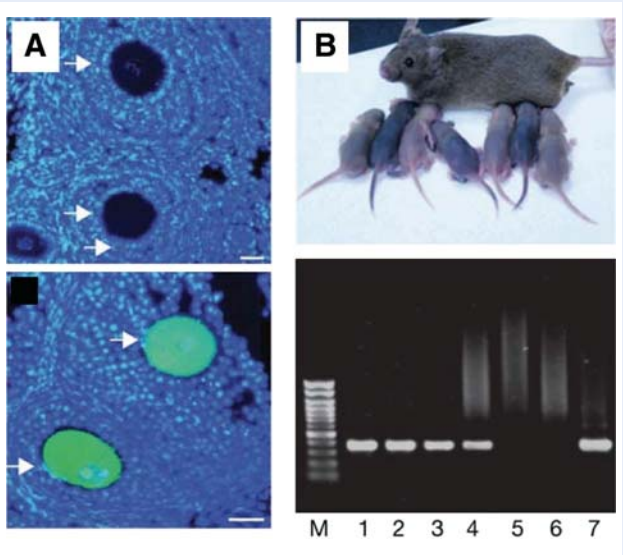

Figure 1 Production of oocytes and offspring after transplantation of labelled FGSC.

(**A**) Zou *et al*. demonstrated that the transplantation of FGSC that expressed GFP into chemotherapy-treated animals led to the formation of follicles containing GFP-positive oocytes (bottom, compare with wild-type control in top panel). (**B**) Animals that received transplants were mated with wild-type males resulting in litters of offspring, approximately one-quarter of which were transgenic for GFP (genotyping shown in bottom panel, lanes 1–4 are transgenic offspring, lanes 5 and 6 are wild-type and lane 7 is a positive control). Images reproduced with permission of the Nature Publishing Group.

FGSC. A quantitative analysis counting GFP-positive oocytes in cell-delivered versus control animals was not provided. Even so, this unambiguous qualitative demonstration that their cell lines were capable of oogenesis *in vivo* led to mating trials to see if these labelled oocytes were capable of supporting fertilization and offspring production.

Mating trials using chemotherapy-conditioned animals that received ovarian injections of GFP-expressing nFGSC (passaged 45 times) or aFGSC (in culture for 15 weeks) were similarly successful. Approximately 80% of animals receiving cell injections produced offspring after mating with a wild-type male (Fig. 1B). Twenty-nine of 108 offspring bore the GFP transgene when nFGSC were used, and 24 of 85 when aFGSC were used.

One might predict that most, if not all offspring produced within these experiments should be derived from GFP-positive FGSC. Two factors make this unlikely. First, the busulfan/cyclophosphamide regimen has been shown to deplete oocytes in a pronounced but gradual fashion over a period of weeks. This allows for several litters to be produced from 'host' oocytes (Lee *et al*., 2007) before their complete eradication. Due to this, the contribution of non-transgenic host oocytes to offspring could be significant. Second, the authors strategy for the establishment of labelled GFP-positive FGSC using retrovirus infection would result in a heterogeneous population of cells available for transplantation. As the authors make no mention of establishing clonal FGSC lines, transplanted cells would be expected to have differing transgene insertion sites and copy numbers. Some fraction of this population would be expected to lose the transgene to either the first or second polar body; in this way, an oocyte derived from a transgenic FGSC could give rise to a wild-type offspring. It is possible that clonal lines of GFP−FGSC with fully characterized transgene insertion site(s) will lead to predictable and increased numbers of transgenic offspring versus the heterogenous population used here. For these reasons, it is possible that the ability of FGSCs to generate offspring is underestimated rather than overestimated.

The origins of FGSCs

Zou *et al*.'s work sets a new standard in the field of oogenesis, where offspring can indeed be produced from oocytes made anew during adult life. The stem cells isolated during these studies have remarkable features, not the least of which is their apparent unipotency, their demonstrated ability to give rise to one type of cell, the oocyte. Important questions remain about the origins of FGSCs and their physiological function *in vivo*. It is reasonable to ask 'where do FGSCs come from?' and 'what exactly do these cells do?'.

If one supposes that FGSCs arise during the specification of PGCs, there are only a few logical explanations for their origins. All of those germ cells that progress through oogonial proliferation, to germline cyst formation and breakdown (Pepling and Spradling, 1998, 2001), and that form primordial follicles are almost certainly disqualified due to their development into (non-proliferative) oocytes. Are FGSCs holdover PGCs, or, are they oogonia that somehow avoid commitment to cyst and follicle formation and arrest in prophase of meiosis I?

Zou *et al*. include some data that sheds some light on these questions. If FGSCs are holdover PGCs, it is reasonable to hypothesize that these cells should behave as embryonic germ cell (EGC) lines [see (Kerr *et al*., 2006) for a review] that can be established from PGCs. Indeed, FGSCs were shown to have similar long-term proliferation potential and gene expression profiles as EGCs. However, the authors show that FGSCs were incapable of development into teratomas when injected into the subcutis of nude mice. This contrast with EGCs that readily develop into teratomas is telling. A recent report demonstrated that 'postmigratory' PGCs, isolated from ovaries as late as embryonic day 13.5 could be established as EGC lines (Shim *et al*., 2008). It is not clear how those authors distinguished between postmigratory PGCs and oogonia, but their results suggest that the proliferative germ cells that can be isolated from fetal ovaries at day 13.5 are either distinct from those isolated by Zou *et al*. in post-natal ovaries, or included them as an unrecognized cell fraction. A timeline and schematic is shown in Fig. 2 to illustrate this point; from this information, we predict that FGSC arises between the border of PGC and oogonia development and the initiation of germline cysts.

We must return to the overarching question: is the production of oocytes by stem cells in mice a normal, physiological process, or, are these cells only relevant to experimental and treatment modalities? Accordingly, if the ovaries of mice contain a number of these cells during adult life, what is their proportional contribution, if any, to the oocyte pool that exists in the ovary at any time? Are FGSCs required to support oocyte numbers in the ovary or are they only stimulated during a crisis? If they can be stimulated to produce new oocytes, why do ovaries cease to function as in the menopause, versus the testis where germ stem cells support sperm production for life? It will take years of clever experimentation to better understand ovarian physiology and what the ovary is truly 'capable of' in terms of its supply of healthy oocytes.

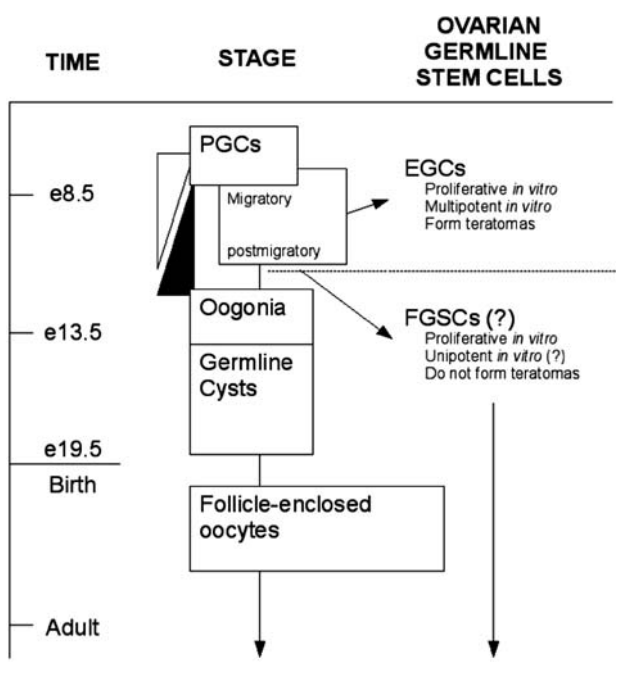

Figure 2 Timing of development of ovarian germline stem cells.

This schematic is an attempt at placing FGSCs as described by Zou *et al*. into developmental context using what is known about PGC- and ovary-derived stem cells. The triangles shown indicate the transition from postmigratory PGCs to oogonia. As EGCs lines with distinct features from FGSCs can be established from both migratory and postmigratory PGCs, it is likely that FGSCs are established or 'set aside' near the boundary of the PGC and oogonia stages. PGC, primordial germ cell; EGC, embryonic germ cell; FGSC, female germline stem cell.

Do FGSCs exist in the human?

The question of whether such cells exist in female humans must now be definitively addressed. This work attracts attention from biologists and the lay public alike, due to hopes that one day healthy babies will result from such technologies. The function of the ovary is of course not limited to offspring production, supporting a myriad of health and wellness parameters in premenopausal women (Perez *et al*., 2007; Pal *et al*., 2008). Thus restored ovarian function using a woman's own FGSC equivalent cells would lead to quality of life improvements in a potentially enormous population of aging women who might seek out such treatment. As mentioned at the outset, ovarian physiology between mammalian species is quite diverse and the strategies used to produce fertilization-competent oocytes can differ greatly. Indeed, mice are not human. However, the conservation of molecular mechanisms that guide germline development, and produce healthy oocytes between the mouse and human is undeniable. The tools that Zou *et al*. used to isolate FGSCs from newborn and adult mice, when validated, should be directly applicable to attempts to isolate similar cells from human ovarian biopsies. Even if germ stem cells do not exist in humans we will still learn much by investigating the question as we improve *in vitro* culture techniques and cell and reagent delivery to human ovarian tissue. If germ stem cells are found to exist during adult life in humans, their potential to be stimulated or used in transplantation regimes to make new oocytes and support ovarian function and fertility is enormous.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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